REPORT DOCUMENTATION PAGE

Form Approved
OMB NO. 0704-0188

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	20 July 05	FINAL: 20 May 02 - 19 Feb 04

4. TITLE AND SUBTITLE
Molecular Analysis of Olfactory Neural Circuits

5. FUNDING NUMBERS
DAAD19-02-1-0170

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8. PERFORMING ORGANIZATION REPORT NUMBER
102566 (FHCRC)

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U. S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER
44033.1-LS

11. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12 a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

12 b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

The olfactory system detects odorants that elicit diverse odor perceptions as well as pheromones that stimulate instinctive behaviors. We previously identified a large family of odorant receptors and determined how signals derived from those receptors are organized in the nose and then the olfactory bulb and cortex. To explore neural circuits in higher brain areas that control olfactory perception and behavior, new technologies are needed. We took the first steps in this direction in this project. First, we showed that a fusion protein containing Cre recombinase, the transfer domain of Diphtheria toxin, and barley lectin (BL), a transneuronal tracer, has Cre recombinase activity, setting the stage for its potential use as a regenerative transneuronal tracer for the visualization of complex neural circuits. Second, we prepared a series of 'inducer' and 'reporter' transgenic mouse lines that, when crossed, may allow the expression of a standard, or regenerative, transneuronal tracer in specific neurons to be controlled by an antibiotic or hormone analog. Third, we showed Cre-dependent, conditional expression of a transneuronal tracer in one of the reporter mouse lines. By crossing this line with mice expressing Cre in neurons with known functions (e.g. appetite), it will now be possible to elucidate neural circuits and genes that control specific functions.

14. SUBJECT TERMS	15. NUMBER OF PAGES
olfactory, neural circuits, perception, behavior, transgenic mice, transneuronal tracer	3
	16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION ON THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

20050822024

FINAL PROGRESS REPORT

Scientific Progress and Accomplishments:

In previous studies, we identified the odorant receptor family, which detects odorous molecules in the nose, and determined how inputs from those receptors are organized in the nose and then in the olfactory bulb. We also began to analyze how signals derived from individual types of odorant receptors are organized in the olfactory cortex. However, new technologies are needed to investigate the neural circuits in higher brain areas that ultimately generate perceptual and behavioral responses to olfactory stimuli. In addition, new methods are needed in order to define neural circuits that control other functions, such as basic drives and behaviors. The major goal of this project was to develop such technologies and, if possible, to use them to investigate olfactory neural circuits.

In mice, odorant detection in the nose is mediated by ~1000 different odorant receptors (ORs), each expressed by a different subset of olfactory sensory neurons (OSNs). Using gene targeting, we had prepared 'knockin' mice in which a transneuronal tracer, barley lectin (BL) was coexpressed with either the M5 or M50 OR gene. Using immunohistochemistry with anti-BL antibodies, we found that BL that was coexpressed with a single OR gene was transferred to a small set of neurons in the olfactory bulb (OB) and then, from those neurons, to third-order neurons in the olfactory cortex (OC).

Analysis of the patterning of labeled neurons seen in the OC of the M5 and M50 knockin mice showed that there is a stereotyped map of OR inputs in the OC. In this map, inputs from a single OR are targeted to clusters of cortical neurons at specific locations that are virtually identical in different individuals and are usually bilaterally symmetrical in the left and right brain. These studies also indicated that inputs from different ORs overlap partially in the OC and that single cortical neurons may receive input from a combination of ORs, possibly allowing an initial integration of each odorant's combinatorial receptor code. These studies further showed that input from the same OR is sent to multiple olfactory cortical areas, an arrangement that may allow inputs from the same ORs to be processed in parallel, possibly in different fashions, prior to transmission to the neocortex and limbic system.

While experiments with the M5 and M50 knockin mice shed light on how information is organized in the OC, the major goal of this project was to develop new techniques that would permit analyses of how olfactory information is organized beyond the OC.

In the course of this project, we made a series of genetically altered mouse lines for the purpose of defining neural circuits that control perceptual and behavioral responses to olfactory stimuli. By coexpressing barley lectin with the M5 or M50 OR gene, we were able to visualize how inputs derived from individual types of ORs in the nose are organized in the cortex. In this project, we asked whether it would be possible to construct a regenerative tracing system. The goal was to find a way that a "regenerative" transneuronal tracer could be expressed in specific neurons and then transferred to their synaptic partners, where the tracer would not only allow visualization of the connected neurons, but also induce the expression of gene encoding itself. This would 'regenerate' the tracer in each neuron in turn in a chain of connected neurons (i.e., a neural circuit), allowing the visualization and thereby the definition of specific neural circuits. If this were feasible, a regenerative tracer could also be used to induce the expression of other genes in the connected neurons, such as genes that would permit isolation of the neurons and analysis of their gene expression patterns.

In initial studies, we prepared a DNA construct that encoded a fusion protein composed of Cre recombinase followed by the transfer domain of Diphtheria toxin followed by the transneuronal tracer, BL. We hypothesized that, when expressed in neurons, the fusion protein (Cre-DT(T)-BL) would be released at synapses and endocytosed by synaptic partners. The DT(T) domain of the protein would then permit transfer of the Cre

domain from endosomes into the cytoplasm, where Cre would be released by reduction of a disulfide bond by which it would be bound to the DT(T) domain. Cre would then enter the nucleus where it would act at loxP recognition sites to effect the excision of selected sequences flanked by loxP sites. We first tested the function of Cre-DT(T)-BL by expressing it in vitro in a cell line along with a Cre-inducible reporter. We next prepared DNA constructs in which the M5 or M50 OR coding region was followed by an IRES (internal ribosome entry site) sequence followed by the Cre-DT(T)-BL coding sequence. Efforts to obtain genetically altered mice in which the endogenous M5 or M50 gene was replaced by the corresponding, genetically-altered allele were unsuccessful, so we subsequently focused on alternative approaches.

While a regenerative tracer could potentially permit elucidation of complex neural circuits, it would be highly advantageous to have temporal control over its initial activation. This would permit examination of labeled neurons at different times following activation of the tracer and thus the placements of the neurons within a circuit relative to one another. To this end, we prepared a series of transgenic mouse lines of two types, 'inducers' and 'reporters'. In these experiments, we focused on GnRH neurons, a small subset of neurons in the hypothalamus that are key regulators of reproductive physiology and behavior and can be influenced by olfactory system signals. We prepared mice carrying three different types of transgenic constructs in which Cre function would, in theory, be induced in GnRH neurons by a hormone or an antibiotic. Selective expression of the transgene in GnRH neurons would be achieved using the promoter of the GnRH gene in the three lines. In each of the lines, Cre expression/function would be temporally (conditionally) controlled by a hormone or antibiotic.

Inducer Lines:

1. Pgnrh-CreER. The GnRH promoter drives the expression of a Cre recombinase-estrogen receptor fusion protein. Administration of 4-hydroxytamoxifen to the mouse and its binding to the ER domain of the fusion protein allows the fusion protein to enter the nucleus and excise a loxP-flanked sequence in the genome.

2. Pgnrh-CrePR. The GnRH promoter controls the expression of a Cre-recombinase-progesterone fusion protein. RU-486 administered to the animal binds to the PR domain of the fusion protein, allowing the protein to enter the nucleus, where the Cre domain can excise a loxP-flanked sequence in the genome.

3. Pgnrh-Cretet. The GnRH promoter controls the expression of RTTA. Doxycycline administered to the animal binds to RTTA allowing it to activate the expression of Cre, which is controlled by the Tet operator.

We also prepared transgenic mice carrying two different types of 'reporters' and used a different reporter mouse line prepared by another group as a control. In these lines, Cre does, or should, allow the expression of a reporter protein.

Reporter Lines:

4. Z/AP. A chicken beta-actin promoter with upstream CMV enhancer elements is followed by a lacZ (Z) gene that is flanked by loxP sites and followed by an alkaline phosphatase (AP) coding sequence (Lobe et al, 1999; obtained as a gift from A. Nagy). In Z/AP mice, lacZ expression is detected in most or all cells, but is replaced by the expression of AP in cells that produce Cre recombinase.

5. Z/BL. This transgene is identical to that of Lobe et al, 1999, except that AP is replaced by BL, the transneuronal tracer.

6. Z/CDB. This transgene is identical to Z/BL, except that the BL is replaced by the experimental 'regenerative tracer', CDB (Cre-DT(T)-BL.

We obtained 3 transgenic founder mouse lines each for the above transgenes #1, 2, and 3 and one each for transgenes # 5 and 6. Some planned crosses between inducer and reporter lines have been completed and some preliminary tests of the inducible lines have been performed in which antibiotic or hormone analogs have been administered to mice. However, further analyses are needed before it is clear whether the inducible lines are functional and the conditions under which induction and Cre recombinase function can be best achieved.

In addition to crossing the above inducer and reporter mouse lines, we have crossed both Z/BL and Z/CDB lines with a mouse line in which the OMP (olfactory marker protein) promoter drives the expression of Cre exclusively in sensory neurons in the olfactory system (OMP-Cre mice, a gift from R. Axel). Studies with these double transgenic mice and the single transgenic Z/BL and Z/CDB mice indicate that Cre-dependent expression of BL occurs from the Z/BL transgene, but not from the Z/CDB transgene and, moreover, that the Z/CDB sequence has acquired at least one mutation. Further analysis of the Z/CDB regenerative tracer system will require the generation of additional mouse lines.

The success obtained with the Z/BL mouse line indicates that this line can now be used to gain insight into a variety of neural circuits that control different functions. By crossing Z/BL mice with mice in which Cre is expressed in neurons involved in different functions, it will be possible to rapidly gain information about the neural circuits in which those neurons are involved. Moreover, using double labeling for BL and other neuronal markers, it will be possible to identify genes that are specifically expressed in neurons that comprise neural circuits with different functions, information that could speed the development of pharmaceutical drugs to treat disorders involving specific functions.

Publications during reporting period:

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Scientific Personnel supported by this project: Mehran Sam; other scientific personnel who participated in the project were supported by other sources. Honors/awards/degrees received: none

Report of inventions: none